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Studies on Carbon Material Requirements for Bacterial Proliferation and Spore Germination under Stress Conditions: a New Mechanism Involving Transmission of Physical Signals

MICHIO MATSUHASHI,[1]* ALLA N. PANKRUSHINA,[1,2] KATSURA ENDOH,[1] HIROSHI WATANABE,[1] YOSHIHIRO MANO,[1] MASAO HYODO,[1] TAKASHI FUJITA,[3] KIYOHIKO KUNUGITA,[3] TOMOHIKO KANEKO,[4] AND SUGIO OTANI[4]

Department of Biological Science and Technology[1] and Department of Material Science and Technology,[4] Tokai University, 317 Nishino, Numazu-shi, Shizuoka-ken 410-03, and Tsukuba Research Institute of Fujisawa Pharmaceutical Company, Tsukuba, Ibaraki-ken,[3] Japan, and Department of Biochemistry and Biotechnology, Tver State University, 170 000 Tver, Jelabova 33, Russia[2]

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The growth of bacteria is often enhanced by addition of carbon materials such as graphite or activated charcoal to the growth medium. In this work, bacterial strains that strictly require such carbon materials under the ordinarily lethal stress caused by high concentrations of salt were isolated. The organisms were gram-positive, spore-forming, sugar-nonfermenting aerobic bacilli and were provisionally designated "*Bacillus carbophilus*" Kasumi after examination of their phenotypic traits. The growth- and germination-promoting effects of graphite and activated charcoal were demonstrated either quantitatively on agar plates containing fine crystals of the carbon materials mixed with a nonpermissive growth medium or qualitatively on agar plates on nonpermissive growth media half-covered with fine carbon particles. Further experiments demonstrated a novel feature of the phenomenon; i.e., the ability to induce colony formation on the nonpermissive plate was transmissible through the air, as well as through plastic or glass barriers. The mechanism probably involves transmission of physical signals regulating cell growth.

The unique biological effects of carbon materials, such as graphite and activated charcoal, have frequently been recognized: e.g., charcoal powders are often applied to the soil to enhance plant growth or used to facilitate the proliferation of bacteria such as *Neisseria gonorrhoeae* (4), *Haemophilus pertussis* (7), and *Legionella pneumophila* (3); artificial cardiac valves made of carbon displayed antithrombogenecity (1, 5); and carbon tooth implants displayed a favorable affinity to gingival tissue (10). It seems to be generally accepted that these carbon materials act indirectly on living organisms, e.g., by adsorbing inhibitory substances from the medium. We report here that carbon exerts a novel effect on the proliferation of at least some bacteria, involving an unexpected mechanism.

MATERIALS AND METHODS

Carbon and other materials. Graphite preparations were provided by the Toyo Carbon Co., Osaka, Japan (graphites A to C), and the Mitsubishi Pencil Co., Tokyo, Japan (graphites 1 to 4). Their sources and properties are listed in Table 1. Two activated-charcoal preparations, one washed with HCl and the other washed with HCl and then neutralized, were Sigma products. Aluminum oxide (Wako Pure Chemicals, Kyoto, Japan) and levigated alumina (Norton Co., Worcester, Mich.) were commercial products. Glass powders were prepared by grinding microscope slide coverslips in a ceramic mortar, washed repeatedly with water, and sterilized by autoclaving. Carbon materials scattered on agar plates were used after exhaustive sterilization by heating at about 700°C for 1 min on a gas burner. In other cases, they were mixed with nutrient agar and autoclaved at 120°C for 15 min.

Bacteria and growth conditions. Isolation and preliminary characterization of bacteria with high graphite requirements are described below. These are *Bacillus* strains and are maintained in our collections (Tokai University and Fujisawa Pharmaceutical Co.) and that of the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (strain stock FERM P-14455). Taxonomic studies of these bacterial species have been completed and will be reported elsewhere (3a). Bacterial cells were usually cultivated in Trypticase soy broth (Baltimore Biological Laboratory; 30 g/liter) or on agar plates containing Trypticase soy broth or Bacto Antibiotic Medium 3 (AM3; Difco; originally 17.5 g/liter) diluted twofold with water (8.75 g/liter; AM3/2). Potassium chloride or sodium chloride was added to AM3/2 in the concentrations indicated below and in the figure legends. The medium was solidified with 1.5% (wt/vol) agar (Wako Pure Chemicals) for plating work. When indicated, 2.5 g of carbon material per liter was mixed with the medium before autoclaving. In most qualitative studies, 5- to 10-μl suspensions of cells or spores (10^3 to 10^4 viable bacteria) were streaked onto the agar plate by reciprocal motion of an inoculation loop. Agar plates containing Trypticase soy broth or AM3/2 and 1% KCl mixed with 2.5 g of graphite C per liter were used for quantitative estimation of colony-forming efficiencies. For this purpose, a 100-μl suspension containing 10^2 to 10^4 fresh vegetative cells or spores in AM3/2 was inoculated onto the plate with a triangle glass spreader stick. Plastic or glass petri dishes (9-cm diameter) were used in the plating work. Culture temperatures and other conditions were as stated below and in the figure legends. In standard experiments, mid-exponential-phase cells (2×10^7 cells per ml as measured on a Trypticase soy agar plate) were prepared by 10% inoculation of an overnight culture in Trypticase soy broth at 30°C into the same fresh medium and incubated with shaking for 2 to 3 h at 30°C. Spores were prepared by maintaining colonies formed on AM3/2 plates at 37°C for 2 days and then for several weeks at room temperature to complete sporulation. Both cells and spores were diluted with AM3/2 and used immediately for plating experiments. In some experiments, spores were diluted with water, providing similar results. Microscopic examination demonstrated that both cells and spores were homogeneous and that the possibility of contamination of one with the other was less than 10^{-2}. The *Bacillus subtilis* used in the experiments was strain JH642 (*pheA1 trpC2*) (9) obtained from F. Kawamura. A mutant of "*Bacillus carbophilus*" Kasumi strain 6 resistant to 200 mg of streptomycin per liter was isolated in this work by spontaneous mutation (Kasumi strain 6S1).

Microscopy. Suspensions of bacterial cells and spores were mounted on a 1-mm-thick layer of saline-agar (0.75% NaCl and 1.5% agar), covered with glass coverslips, and examined under a phase-contrast microscope (Nikon Apophoto) at a magnification of ×600.

* Corresponding author. Mailing address: Department of Biological Science and Technology, Tokai University, 317 Nishino, Numazu-shi, Shizuoka-ken 410-03, Japan. Phone: (81.559) 68-1111, ext. 2102. Fax: (81.559) 68-1156.

TABLE 1. Sources and properties of graphite used

Graphite	Source	Form	Diam (μm)
1	Natural graphite from China		1
2	Natural graphite from Sri Lanka		10
3	Synthetic graphite	Single crystal	25
4	Natural graphite from Sri Lanka	Large scale	
A	Synthetic graphite	Polycrystals	21
B	Synthetic graphite	Polycrystals	10
C	Coke heated at 1,400°C	Polycrystals (turbostractic structure)	18

RESULTS

Isolation of bacterial strains requiring graphite for colony formation. Bacterial strains that displayed strong requirements for graphite on a salt stress plate of AM3 agar or of AM3/2 agar supplemented with 1% (wt/vol) KCl at 44°C were isolated. These strains grew on the above-described plates only when graphite was mixed with the medium or fine crystals of graphite were placed on the plates. Seven such strains were subjected to systematic taxonomic examination. They were characterized as *Bacillus* species in view of their positive Gram staining, long rod shape, formation of a single ellipsoidal spore at one terminus of the cell, aerobic growth, lack of acid formation in sugar fermentation, about 38% GC content, murein type containing *meso*-diaminopimelate, and other traits. All the strains, which we provisionally refer to as "*B. carbophilus*" Kasumi strains (see Materials and Methods), displayed similar phenotypes but had diverse sensitivities to high salt concentrations. Graphite requirements were observed for colony formation by both cells and spores.

Figures 1 and 2 show examples of Kasumi strain 6 forming colonies from inoculated cells and spores, respectively, in the presence of graphite. These figures also show that growing cells are more fragile than spores under salt stress conditions. When graphite crystals were placed on the plate (right half of each plate) directly after inoculation of the cells, colonies were rapidly formed around the graphite crystals (Fig. 1A). However, when the inoculated cells were incubated for 1 day (Fig. 1B) or 2 days (Fig. 1C) before the graphite crystals were applied, no colonies were formed after further incubation of the plates. On the other hand, spores are more resistant to salt stress. Application of graphite after 1 day (Fig. 2B), 2 days (Fig. 2C), or even 7 days (photograph not shown) of incubation also induced colony formation. No colonies were formed when graphite was totally absent from the inoculated plate (photographs not shown). The graphite dependency of colony formation was more evident at 44°C than at 30 or 37°C.

Different graphite preparations, i.e., graphites A, B, C, 1, 2, 3, and 4 (Table 1), displayed similar growth-promoting effects on "*B. carbophilus*" Kasumi strains (Kasumi strains 4 and 6 having been tested most extensively), as did an HCl-washed preparation and an HCl-washed and neutralized preparation of activated charcoal under similar growth conditions. Neither aluminum oxide preparations nor glass powders had any appreciable effect (photographs not shown).

AM3 itself contains 1.13% (wt/vol) KCl. AM3/2 (0.6% KCl) permitted growth at 44°C, but addition of 0.5% KCl inhibited growth unless graphite was also added. NaCl added to AM3/2 manifested effects similar to but slightly less intense than those of KCl (photograph not shown).

To quantitate the graphite requirement, agar plates containing AM3/2 with 1% added KCl and 0.25% fine crystalline graphite C were prepared. Colony-forming efficiency on both the plates containing and not containing graphite was estimated and compared with that on Trypticase soy agar plates, on which the highest colony-forming efficiency for Kasumi strains in the absence of graphite has so far been obtained. (The plating efficiencies did not increase when 0.25% graphite C was added to the Trypticase soy agar plate). The observed plating efficiencies of growing cells and spores of strain 6 are shown in Table 2. Neither cells nor spores displayed any measurable growth on the AM3/2 agar plates containing 1% added KCl. Colony-forming efficiencies on the AM3/2 agar plate containing added KCl and graphite C were about 20% for growing

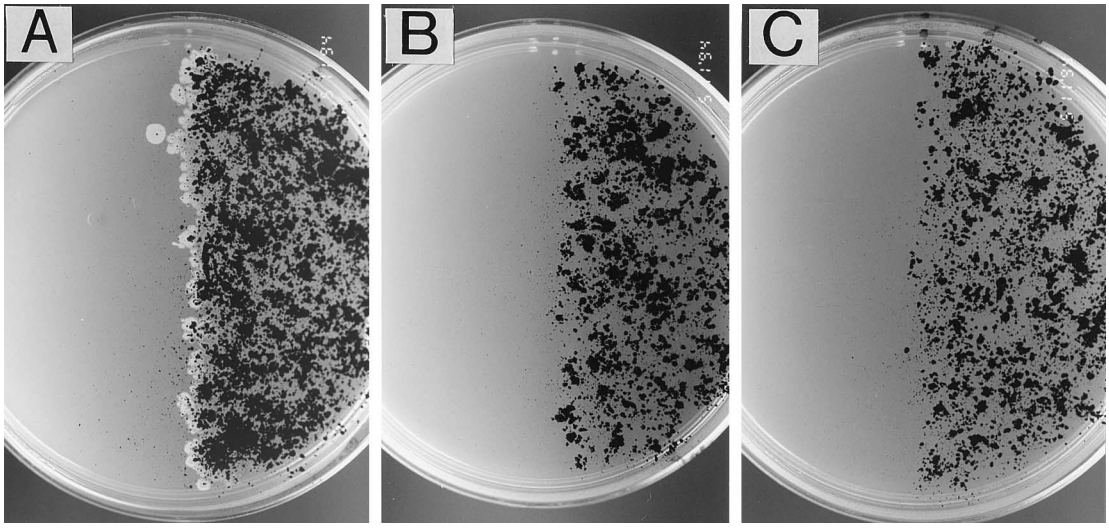

FIG. 1. Graphite-dependent growth of fresh cells of "*B. carbophilus*" Kasumi strain 6 on plates under high-salt stress. Vegetative bacteria (17,000 viable cells) were inoculated on AM3/2 agar plates containing 1% added KCl by using a platinum inoculation loop with reciprocal horizontal motion to form a gradient of viable counts from the top of the plate to the bottom. Plates were incubated for 4 days at 44°C in an incubator. The right halves of plates were covered with sterilized graphite B immediately (A), 1 day (B), or 2 days (C) after inoculation. The photographs were taken with transmitted illumination.

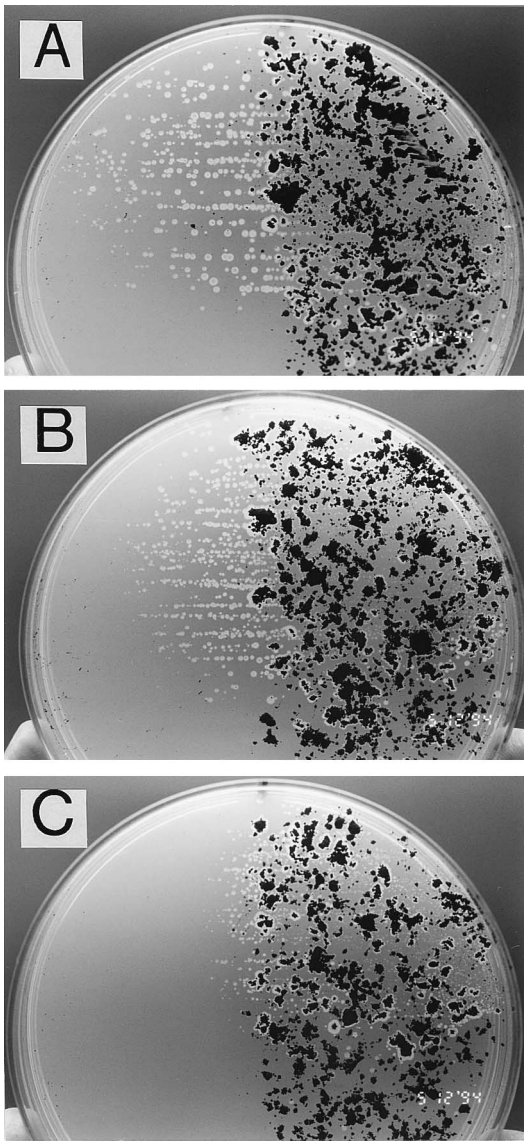

FIG. 2. Graphite-dependent growth of spores of "*B. carbophilus*" Kasumi strain 6 on plates under high-salt stress. Spores (10^4 viable bacteria) were inoculated. Other experimental conditions were the same as described for Fig. 1.

cells and about 90% for spores, as compared with those on the Trypticase soy agar plate. Similar results were obtained with cells and spores of Kasumi strain 4.

Colony-forming effects of graphite are long range. As shown in Fig. 2, when spores of Kasumi strain 6 were inoculated on an ordinarily nonpermissive plate and half of the plate was covered with graphite crystals, colonies formed overnight directly around the graphite crystals (photograph not shown, but similar to Fig. 2C) and, upon prolonged incubation, extended from the area of the graphite nearly to the other side of the plate (Fig. 2A). On the other hand, when exponentially growing cells were inoculated on an ordinarily nonpermissive plate and half of the plate was covered with graphite, colonies were formed around the graphite crystals but did not extend during further incubation (Fig. 1A). The effect of graphite on spores might reflect a long-range phenomenon or trace contamination of the left sides of the plates by invisible graphite crystals, although this latter possibility seems unlikely (see the experiment with cells, Fig. 1).

TABLE 2. Colony-forming efficiencies of "*B. carbophilus*" Kasumi strain 6 on different media

Bacterial stage	Plate composition	Titer	Relative plating efficiency
Vegetative[a]	Trypticase soy broth	8.1×10^7	1.0
	AM3/2 + 1% (wt/vol) KCl	$<10^3$	$<10^{-5}$
	AM3/2 + 1% KCl + 0.25% (wt/vol) graphite C	1.7×10^7	0.21
Spore[b]	Trypticase soy broth	8.45×10^6	1.0
	AM3/2 + 1% KCl	$<10^3$	$<10^{-5}$
	AM3/2 + 1% KCl + 0.25% graphite C	7.6×10^6	0.90

[a] Colonies freshly cultured on Trypticase soy agar plates at 44°C (6 h) were suspended in AM3/2 to prepare a suspension with an A_{660} of 0.05 in a test tube (1.8 cm thick) measured in ICM photometer (Hillsboro, Oreg.). No spores were visible under a phase-contrast microscope. The suspension was diluted 10, 50, 250, and 1,250 times with AM3/2, and 100-μl aliquots of each dilution were inoculated onto agar plates as indicated. The number of colonies was counted after incubation at 44°C for 1 day (Trypticase soy broth) or 2 days (AM3/2 with supplements). The colony formation on AM3/2 plates with supplements was complete in 2 days incubation at 44°C. Similar results were obtained with cells cultured freshly in Trypticase soy broth at 30°C as described in Materials and Methods (data not shown).

[b] Spores (see Materials and Methods) were suspended in sterilized deionized water to an A_{660} of 0.05; the suspension was diluted 10, 50, 250, and 1,250 times with sterilized deionized water; and 100-μl aliquots of each dilution were inoculated onto appropriate plates (experiment 1). Plates were incubated for 3 days (Trypticase soy broth) or 5 days (AM3/2 with supplements). The colony formation on the AM3/2 plates with supplements was complete in 4 days of incubation at 44°C. No vegetative cells were visible in the inoculating spore suspension under a phase-contrast microscope. Experiments were performed by different persons with similar results.

The colony-forming effects of graphite are transmissible through the air and through plastic or glass barriers. To distinguish between these possibilities and to ascertain whether a substance diffusing through the agar was responsible for the effect, we prepared separately two semidisks containing AM3/2 and 1% added KCl. One of the semidisks also contained fine crystals of graphite. The nonpermissive half-plate was inoculated with spores of Kasumi strain 6 by using an inoculation loop with a reciprocal horizontal motion, thereby creating a gradient of inoculum size from the top of the plate to the bottom (see Fig. 3). The semidisks were placed side by side in a petri dish with an intervening gap of several millimeters. The graphite-containing plate was either profusely inoculated with cells and spores of Kasumi strain 4 (Fig. 3A) or not inoculated (Fig. 3B). Upon incubation, bacterial colonies grew first on the graphite-containing agar half-plate as a thin semitransparent film of cells, and then colony formation started on the clear plate across the gap, progressively extending from the area close to the graphite plate further toward the other side (Fig. 3A). No colonies were formed on the nonpermissive plate when the graphite-containing plate was not inoculated (Fig. 3B). The results were similar when Kasumi strain 6 was used to inoculate the graphite agar.

This long-range colony-forming effect was also demonstrable when a barrier of plastic or glass divided the two agar semidisks.

Plastic petri dishes divided into two equal compartments by a septum were used for the experiment displayed in Fig. 4. In one of the compartments was a nonpermissive agar containing AM3/2 with 1% added KCl, and in the other was a graphite-

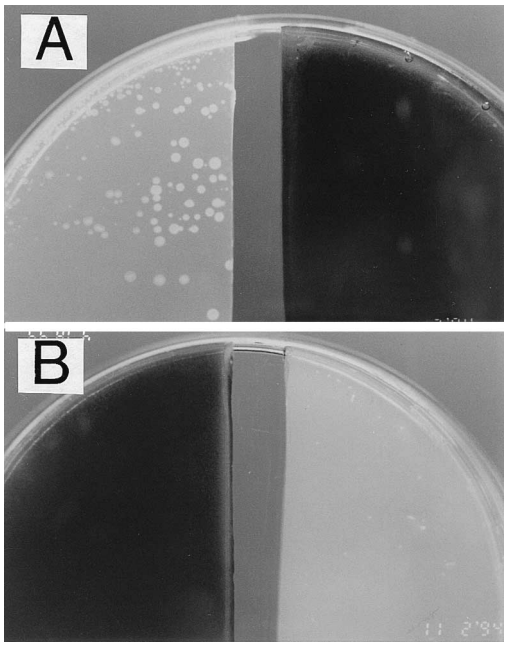

FIG. 3. Long-range colony-forming effect on distantly located spores across a gap. An agar plate containing AM3/2 and 1% added KCl was inoculated with 10 μl of spore suspension (about 10^4 viable spores) by reciprocal horizontal streaking with a platinum loop, and after drying, the agar was cut with the sharp edge of a sterilized knife into two symmetrical semicircular parts. Each of the two semicircles was placed in a new sterilized plastic petri dish, and an uninoculated semicircle containing AM3/2, 1% added KCl, and 0.25% graphite C was placed alongside each inoculated agar semicircle. A strip of agar was removed from each semicircle to form an intervening gap 0.8 to 10 mm in width. In panel A, the graphite-containing agar was then inoculated with about 10^4 cells of "*B. carbophilus*" Kasumi strain 6. Plates were incubated at 44°C for 3 days. The photographs were taken with transmitted illumination.

containing agar as described above. The graphite-containing agar was uniformly inoculated with *B. subtilis* JH642 (Fig. 4A) or "*B. carbophilus*" Kasumi strain 4 or 6 (photograph not shown) or not inoculated (Fig. 4B). After incubation at 44°C for 1 day, colonies were formed all over the graphite-containing agar. Then the nonpermissive agar was inoculated with spores or cells of Kasumi strains 4 and 6, and the plates were further incubated. Colony formation in the nonpermissive plates started from the area close to the dividing plastic wall, but no colonies were formed in the nonpermissive plate when bacterial cells were not inoculated on the graphite-containing plate. The efficiencies were estimated to be about 10% for inoculated Kasumi 4 cells and 5% for inoculated Kasumi 6 cells.

The colonies of Kasumi strains formed on the nonpermissive agar were identified by showing the lack of colony-forming ability on M9 agar supplemented with 50 mg (each) of phenylalanine and tryptophan per liter, on which *B. subtilis* JH642 would grow, and by the yellow-white semitransparent appearance of colonies characteristic of Kasumi strains. Moreover, when a streptomycin-resistant mutant strain, Kasumi strain 6S1, was used to inoculate the nonpermissive agar, the colonies that formed were resistant to streptomycin.

Similar results were obtained when spores of Kasumi strains 4 and 6 were inoculated on the nonpermissive plate.

These results suggest that the cells of *B. subtilis* and "*B. carbophilus*" Kasumi strains growing on the graphite-containing plate transmitted a signal through or above a 1.2-mm plastic barrier to cells of the Kasumi strains, allowing them to grow. A similar effect was seen with divided glass petri dishes.

In order to exclude the possibility of contamination of growth-regulating materials through the air, two covered petri dishes were used to demonstrate the apparent signal transmission.

The first petri dish contained AM3/2-graphite agar and was pregrown with *B. subtilis* or "*B. carbophilus*" Kasumi strain 6 for 1 day. The second petri dish (nonpermissive) contained AM3/2–1% KCl agar and was inoculated with spores of Kasumi strain 6. The efficiency of colony formation by the cells on the nonpermissive plate, inverted and placed directly above the permissive plate (cover to cover), was higher than 50% when the permissive plate was inoculated with *B. subtilis* (Fig. 5A). The efficiency was much lower when "*B. carbophilus*" was used to inoculate the permissive plate (Fig. 5B). No colonies were formed on the nonpermissive plate when the graphite plate was not inoculated (Fig. 5C).

Important aspects of the experiments in this paper were

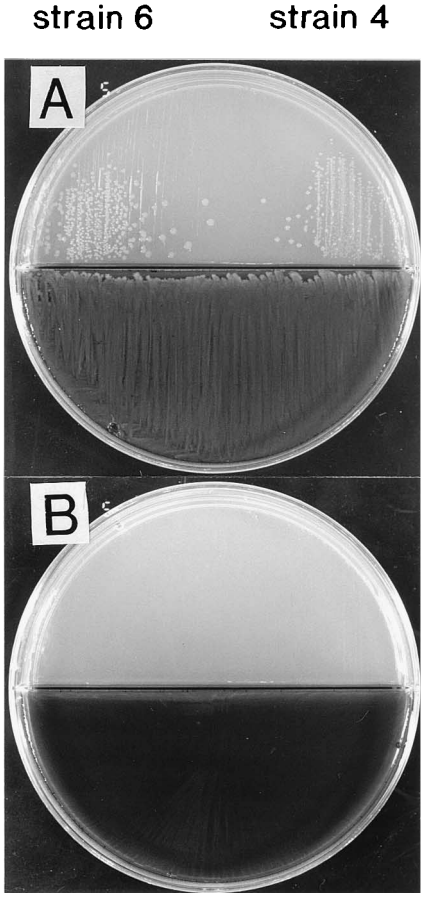

FIG. 4. Transmission of colony-forming effects through a plastic wall: sustenance of cell growth. A plastic petri dish centrally divided into two symmetric compartments by a 1.2-mm plastic wall (Falcon polystyrene petri dish [100 by 15 mm] with two compartments) was used to prepare two semidisk-shaped agar plates completely separated by a plastic wall. One of the two symmetrical half plates contained AM3/2 and 1% added KCl (nonpermissive plate), and other contained AM3/2, 1% added KCl, and 0.25% graphite C (graphite-containing plate). Graphite-containing plates were inoculated with about 10^7 cells of *B. subtilis* (A) or not inoculated (B), and petri dishes were incubated at 37°C for 1 day prior to inoculation of cells on the nonpermissive plate. On the following day, 10^4 cells of "*B. carbophilus*" Kasumi strains 4 (right half of each nonpermissive plate) and 6 (left half of each nonpermissive plate) were inoculated by using a platinum loop with reciprocal perpendicular motion to create a gradient of population toward the center of semidisk plate, and petri dishes were incubated at 44°C for 2 days. The photographs were taken with normal illumination.

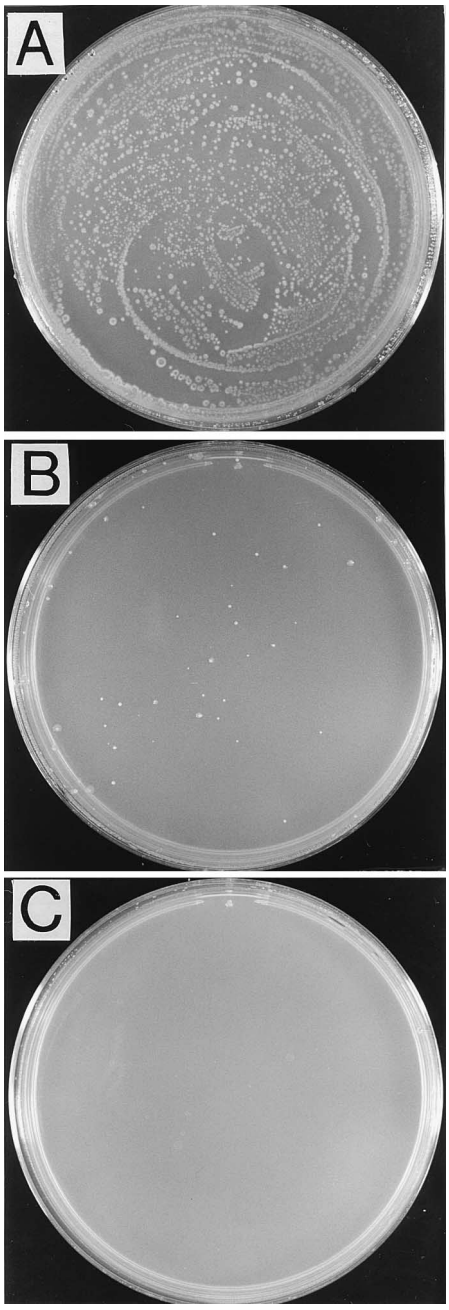

FIG. 5. Transmission of colony-forming effects through a plastic wall: transmission from one covered petri dish to another. Signal transmitter cells were inoculated on agar plates containing AM3/2 and 0.25% graphite B ([A] *B. subtilis*; [B] "*B. carbophilus*" Kasumi strain 6) or not inoculated (C). These plates were preincubated for 1 day. Signal recipient bacteria (about 3,000 viable spores of "*B. carbophilus*" Kasumi strain 6) were inoculated on agar plates containing AM3/2 with 1% added KCl. Petri dishes were stacked cover to cover, i.e., the dish containing the signal recipient cells was inverted and placed above the dish containing the signal transmitter cells. (It mattered little which plate was on top.) The whole system was placed in a cylindrical acrylic box, 3 mm thick, 15 cm wide, and 10 cm high, and incubated at 44°C for 2 days. Encasement of the petri dishes in an acrylic box strongly enhanced colony formation on the nonpermissive plate when signal transmitter cells were present on the other plate, indicating perhaps that reflection of a signal augments its effect. The photographs were taken with normal illumination.

repeated by independent scientists in geographically different institutions, and our results were confirmed. F. Kawamura and H. Yoshikawa in the University of Tokyo carried out experiments with divided petri dishes using spores of "*B. carbophilus*" Kasumi strain 6 as the signal recipient and "*B. carbophilus*" Kasumi strain 6 and *B. subtilis* as signal transmitters and an experiment similar to that of Fig. 5 using *B. subtilis* as the signal transmitter; S. Mizushima in Tokyo College of Pharmacy also carried out similar experiments using *B. subtilis* as the signal transmitter (5a, 7a).

DISCUSSION

The results presented in this paper demonstrate the following: (i) carbon materials such as graphite and activated charcoal permit the growth of "*B. carbophilus*" Kasumi strains under high-salt, high-temperature stress conditions; (ii) once the cells have grown around the carbon material, they transmit colony-forming potential to cells and spores in distant locations; and (iii) transmission of this potential is not interrupted by barriers of plastic or glass, suggesting that transmission is effected by some physical signal. From these results, we may conclude that growth-promoting effects of the carbon materials involve two steps; i.e., first, the carbon material interacts with cells or spores to cause initial formation of neighboring colonies, and then long-range signal transmission induces formation of colonies in distant areas, even in nonpermissive media.

The mechanism of the first step is not known. Probably, the carbon material adsorbs cells and some growth-regulatory substance(s) onto its surface. In the second step, the primary colonies, in conjunction with the carbon material, may transmit signals to distant cells, allowing them to grow under nonpermissive conditions. When graphite was not included, the long-range signal could still be transmitted (unpublished data). However, signal transmission by *B. subtilis* appeared to be more effective in the presence of graphite than in its absence, whereas the growth of *B. subtilis* signal transmitter cells in AM3 or AM3/2 was not increased by the presence of graphite.

As the "*B. carbophilus*" Kasumi strains are spore forming, the experiments of the present work showed that the growth-promoting effects of carbon materials are applicable to both spores and vegetative cells. *Escherichia coli*, which does not form spores, was also found to both transmit and receive the above-described signals under KCl stress (unpublished data).

The mechanism of regulating the process of cell growth and division in bacteria has been investigated precisely, and many of the enzymes, proteins, and genes involved in this mechanism are known (2, 6). Many chemical substances inhibit or activate the process. They influence the process mostly by contact with the cell after being transported through media. Irradiation with UV light, X rays, and beta and gamma rays is known to cause damage to cells, and magnetic fields also appear to display some influence on cell growth (8).

The phenomenon that we have found appears to be totally new. The signal that crosses from one petri dish to another must be of a physical nature, since chemical factors cannot be transmitted through plastic or glass barriers and heat transmission should not be a factor in a thermostatic incubator. Moreover, our recent experiments suggest that equivalent signals can be generated by a sonic device (unpublished data).

The mechanism of this effect and the stimulatory role of carbon in signal transmission could be investigated precisely by studying mutants that are insensitive or supersensitive to the carbon materials and to the transmitted signals.

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